PATENT
Attorney Docket No.: 20695C-008700US
Client Reference No. PL-279.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 9454
Varadi *et al.*	Examiner: Rosanne Kosson
Application No.: 10/816,099	Technology Center/Art Unit: 1653
Filed: March 31, 2004	DECLARATION OF DR. PETER TURECEK UNDER 37 C.F.R. § 1.132
For: KITS FOR MEASURING THROMBIN GENERATION	
Customer No.: 44183	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I, Peter Turecek, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

2. All statements herein made of my own knowledge are true, and statements ma de on information or belief are believed to be true and correct.

3. I am a named inventor on the above-referenced patent application and I previously submitted a Declaration under 37 C.F.R. § 1.132 in support of the patentability of this application over various references cited in an obviousness rejection. The Declaration presented results from experiments demonstrating that (1) the addition of $CaCl_2$ to a fluorescent substrate in an aqueous solution leads to formation of a precipitate; and (2) dissolution of the fluorescent

substrate in an aqueous solution leads to the formation of a fine precipitate. It is my understanding that the obviousness rejection has been maintained.

4. The Examiner notes that concentrations, molar ratios and temperatures, which are parameters that influence solubility, are not provided in the previous Declaration and therefore concludes that the Declaration does not provided sufficient data to show that the mixtures of fluorogenic thrombin substrate and $CaCl_2$ described in the prior art would be expected to precipitate. The Examiner also alleges that the Declaration is insufficient because there are no experiments in which solutions of $CaCl_2$ and ZGGR-AMC are lyophilized separately and rehydrated to form aqueous solutions using varying concentration. In addition, the Examiner alleges that it was known in the prior art that ZGGR-AMC and $CaCl_2$ could be combined to make a clear solution. The Examiner further alleges that the thrombin substrate and $CaCl_2$ are both soluble in water.

5. This Declaration is provided to supply additional information relating to the experiments presented in my previous Declaration and additional data from those sets of experiments. The present Declaration provides additional explanation that fluorogenic thrombin substrates are poorly soluble in water, and that in particular, the fluorogenic substrates in Váradi *et al.* (*J. Thrombosis and Haemostasis* 1:2374-2380, 2003) have very poor solubility in aqueous solutions. Last, this Declaration explains that the combination of thrombin substrate and $CaCl_2$ for the assay described in Váradi *et al.*, typically leads to a precipitate during preparation, even though the precipitate is not discussed in the publication.

6. In the first set of experiments described in my previous Declaration, small amounts (25 mg) of the fluorescent substrate ZGGR-AMC.HCl in powder form were dissolved in 7.4 ml of HNa-DMSO 10% solution with stirring at room temperature. HNa-DMSO is an organic (i.e., non-aqueous) buffer that contains 10% DMSO, 25 mM HEPES and 175 mM NaCl, pH 7.35). ZGGR-AMC is not soluble in an aqueous buffer. After stirring until the substrate was fully dissolved, a 0.58 ml volume of 1 M $CaCl_2$ was added. A precipitate was observed. This disappeared after stirring. However, after 30 minutes standing at room temperature, the solution was cloudy. After shaking, a distinct, fine precipitate was noted. After one hour at 37°C, the

solution cleared. The absorbance values at 405 nm at these various points were measured. This experiment is described under Experiment 1 on page 1 of the attachment to this Declaration ("the Attachment"). The absorbance values are provided in Table 1. The wells B6 through F6 show the absorbance after the addition of $CaCl_2$. The wells B5 through E5 show the substrate before the addition of $CaCl_2$. Figure 1 of the Attachment shows the different forms of the precipitates during the experiments.

Similar observations were observed in a set of experiment using three samples. The experiment as described above was repeated in triplicate and the absorbance was measured (Experiment 2, page 3 of the Attachment). Table 2 shows the absorbance before the addition of $CaCl_2$. After 0.55 ml of 1 M $CaCl_2$ was added to the dissolved substrate (6.8 ml of substrate), the solution became cloudy. This disappeared after stirring at room temperature. After 30 minutes standing at the bench, two of the three samples became cloudy again. The absorbance is shown in Table 3. Altogether, three of the four samples became cloudy in these experiments using small amounts of the substrate.

7. A second set of experiments was performed using full vials of 250 mg of ZGGR-AMC (Experiments 3 and 4, pages 4 and 5 of the Attachment). In Experiment 3, the substrate was dissolved in 74 ml of HNa-DMSO 10% solution and then 6 ml of 1 M $CaCl_2$ was added to the fully dissolved substrate. A precipitate formed. The solution was warmed at 37° for 15 minutes and the precipitate was dissolved with stirring for 45 minutes at room temperature. The solution then sat at room temperature for 2-3 hours. Visual inspection showed that the solution became cloudy over time. This was confirmed by the absorbance measurements, which are summarized in Table 4. Well C5 is the solution buffer without substrate; C6 is after the substrate was dissolved in the DMSO-buffer solution, C7 is after the addition of $CaCl_2$ and re-dissolving of the cloudy precipitate, D2-D11 are aliquots taken after 2 hours of further storing of the vials at room temperature.

This experiment was repeated (Experiment 4). The solution again became cloudy. Absorbance measurements are shown in Table 5. The time was between 30 to 60 minutes. The development of the precipitate is documented by a series of photographs (Figure 2 of the Attachment).

8. Thus, in the foregoing experiments, there was only one instance in which the solution did not become cloudy out of six instances where the $CaCl_2$ was added to the dissolved substrate. The final desired concentrations (5 mM substrate and 75 mM $CaCl_2$) of the working solutions in these experiments were higher than the concentrations in the final assay conditions used in Váradi *et al.* This higher concentration was required for preparing a working reagent, which has to be added to a reaction mixture of a plasma sample and trigger, to achieve a final concentration of reagents as described in Váradi *et al.*

9. Váradi *et al.* is my work. Váradi *et al.* does not detail the preparation of the thrombin substrate/$CaCl_2$ solution described on page 2375 at the second full paragraph. However, for the experiments performed in Varadi *et al.*, the fluorgenic ZGGR-AMC substrate (250 mg) was first dissolved in 74 ml of a HEPES-NaCl buffer containing 10 % DMSO. Warming and vigorous shaking was required to dissolve the precipitate formed upon the addition of the required amounts (6 ml) of 1 M $CaCl_2$ to the dissolved ZGGR-AMC substrate. This solution had a concentration of 75mM $CaCl_2$ and 5 mM thrombin substrate. Once the precipitate was dissolved, the solution was aliquoted and stored frozen at –20°C. For use, an aliquot was thawed, diluted 5-fold with HEPES-NaCl buffer for use in the reaction mixture, resulting in the final concentration of 15 mM $CaCl_2$ and 1 mM thrombin substrate.

10. Fluorescent substrates are usually not water soluble and after lyophilization an organic solvent such as DMSO is required to re-dissolve the substrate, which is not convenient in a clinical environment. For example, according to the manufacturer, the commercially available ZGGR-AMC substrate has to be dissolved in an organic solution. Lawson *et al.* also describes fluorogenic thrombin substrates, which were first dissolved in DMSO to a stock concentration of 10 mM.

As further evidence of the poor solubility of the fluorescent substrate, we tried to reconstitute ZGGR-AMC (250 mg) in water (74 ml) without DMSO, but the powder could not be fully dissolved. We proceeded with adding $CaCl_2$, even though the substrate was not fully dissolved. A fine precipitate was formed. This could not be solubilized, even after heating to 37°C and stirring for 60 minutes (Experiment 5 and Figure 3).

11. A basic requirement for reagent kits is that the kit be "ready to use" and immediately available, if needed. Therefore a reagent in which a precipitate is likely to form (either immediately or delayed) upon the addition of $CaCl_2$, to the substrate is not an acceptable diagnostic reagent. The current invention provides a lyophilized reagent comprising $CaCl_2$ and a thrombin substrate comprising a fluorescent label. The lyophilized mixture is reconstituted prior to use in an aqueous buffer. The DMSO present in the initial buffer to solubilize the fluorescent thrombin substrate is lost during lyophilization. We discovered that no precipitate forms when the lyophilized mixture is dissolved in the aqueous buffer such that the reagent is "ready to use". Organic solvent is not required. Based on my experience in field working with fluorogenic substrates, this was an unexpected and surprising finding.

12. The Declarant has nothing further to say.

Date: _____ By: _____
Peter Turecek, Ph.D.

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Attachment to Declaration

Fluorescence Substrate for TGA Protocol of Dissolving Experiment

Preliminary experiments with small amounts of substrate

Experiment 1

- 25 mg fluorescence substrate powder was weighed in a 10 ml glass vial and dissolved in 7.4 ml of HNa-DMSO10%-solution under stirring at room temperature (magnetic stirrer) until the substrate was fully dissolved. An aliquot of the sample was measured at absorbance of 405 nm – plate well E6 (Table 1)
- After the measurement 0.58 ml of 1M $CaCl_2$-solution was added (at room temperature) to the dissolved substrate → opal fog (cloud) formation has been observed, which disappeared after stirring at room temperature (Absorbance measured in well D6) (Table 1)
- The solution was standing at room temperature at the bench and after 30 minutes the solution became opal, both in the vial and in the well of the micro titer-plate. (Absorbance measured in welsl D6, F6) (Table 1)
- The solution in the vial became clear after shaking at room temperature, with forming of distinct small precipitates. (Absorbance measured in well C6) (Table 1)
- The solution was standing at 37°C for 1 hour and became clear (Absorbance measured in well B6) (Table 1)

Table 1. Absorbance of fluorescence substrate solution before and after the addition of $CaCl_2$

well	Abs 405nm		sample description
E5 - E6	0.048	0.047	Substrate + HNaDMSO10%
D5 - D6	0.048	0.047	immediately after adding $CaCl_2$
D5 - D6	0.048	0.454	Substrate + $CaCl_2$ ~ 30 min RT in micro titer-plate
F5 – F6	0.048	0.264	Substrate + $CaCl_2$ ~ 30 min RT in vial
C5 – C6	0.047	0.092	Substrate + $CaCl_2$ ~ 30 min RT after shaking in vial
B5 – B6	0.048	0.053	Substrate + $CaCl_2$ 1 h 37°C (in vial)

The different phases of the experiment were photographed and shown in Figure 1.

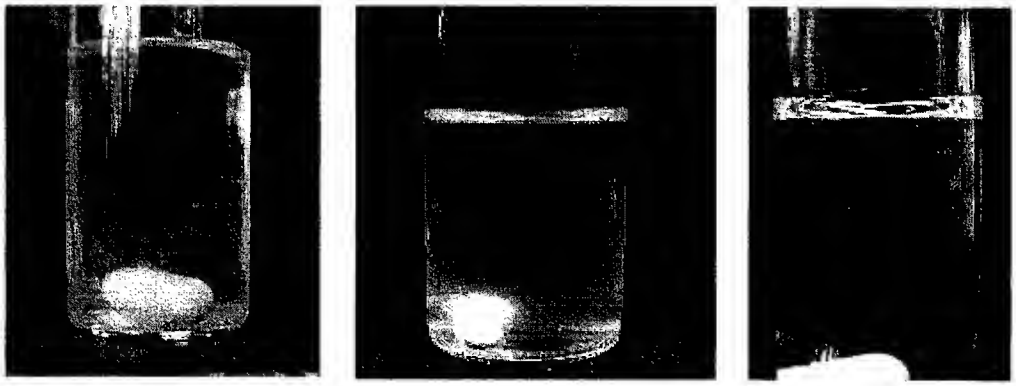
Fluorescence Substrate for TGA **Protocol of Dissolving Experiment**

Preliminary experiments with small amounts of substrate

Figure 1- Precipitate formation in TGA-Substrate dissolving experiment, small amount

- 25 mg Substrate-powder weighed in a vial was dissolved in 7.4 ml HNaDMSO10%-solution (solution is clear)

- After addition of 0.58 ml 1M CaCl2-solution an opal fog formation has been observed, which disappeared after stirring.
- After 30 – 60 minutes standing at room temperature on the bench the substrate / CaCl2-solution becomes opal.
- The opal solution became clear after shaking, however with forming of distinct small precipitates



Experiment 2 (= Experiment 1, but with 3 aliquots)

- 3 x 25 mg fluorescence substrate powder was weighed in each 10 ml glass vial and dissolved in 7.4 ml of HNa-DMSO10%-solution under stirring at room temperature (magnetic stirrer) until the substrate was fully dissolved. (Table 2)
-

Table 2. Absorbance of Fluorescence substrate solution before the addition of $CaCl_2$

well	Abs 405nm		sample description
B2, B11	0.048	0.048	Blank (empty plate)
B3, B10	0.038	0.048	Blank (HNaDMSO-buffer)
B4 - B5	0.043	0.044	sample 1
B6 - B7	0.044	0.043	sample 2
B8 - B9	0.044	0.057	sample 3

- After the measurement 0.55 ml of 1M $CaCl_2$-solution was added to the dissolved Substrate (6.8 ml after sample taking for absorbance measurement) → opal fog (cloud) formation has been observed, which disappeared after stirring at room temperature.
- After 30 minutes standing at the bench (room temperature) the solution remained clear, but 2 of the 3 samples became opal in the micro titer-plate. (Table 3, Absorbance at 405 measured in wells D4-D9). No changes observed in the wells, contained the samples before the addition of $CaCl_2$.

Table 3. Absorbance of Fluorescence substrate solution 30 minutes after the addition of $CaCl_2$

well	Abs 405nm		sample description
D4 – D5	0.050	0.047	sample 1
D6 – D7	0.215	0.216	sample 2
D8 – D9	0.322	0.273	sample 3

Experiments with full vials of substrate

Experiment 3

- 1 vial of fluorescence substrate powder (250 mg) was dissolved in 74 ml HNaDMSO10%-solution under stirring (magnetic stirrer) at room temperature. (Documentation with photo and measurement of the Absorbance at 405 nm - well C6) (Table 4)
- After the substrate was fully dissolved 6 ml of 1M $CaCl_2$-solution was added (at room temperature). White cloudy precipitate was visible (Documentation with photo and measurement of the Absorbance at 405 nm –well C7)
- The solution was warmed up for 15 min at 37°C and dissolved with following stirring for 45 min at room temperature (Documentation with a photo of the clear solution.)
- Absorbance measurement of the solution at 405 nm → after a standing time of 2 hours at the bench the solution became opalescent, which did not disappear even after 3 hours (Absorbance measurement at 405 nm - wells D2-D11).

The absorbance measurement are summarized in Table 4.

Table 4. Absorbance of the dissolved substrate at different time-points

Results of Absorbance measurement					
	Abs 405nm				
well			2 hours	3 hours	sample description
C5	0.038	0.038	0.038	0.040	HNaDMSO10%-Buffer
C6	0.043	0.043	0.044	0.044	Substrate + HNaDMSO10%-Buffer
C7		0.042	0.118	0.209	Substrate + HNaDMSO10%-Buffer with $CaCl_2$ stored in the plate
D2			0.046	0.221	Substrate + HNaDMSO10%-Buffer with $CaCl_2$ 2 hours: sub-sampled from the vial 3 hours: stored in the plate
D3			0.052	0.215	
D4			0.066	0.225	
D5			0.117	0.231	
D6			0.167	0.241	
D7			0.136	0.236	
D8			0.076	0.223	
D9			0.057	0.215	
D10			0.048	0.190	
D11			0.046	0.195	
E2 – F11		~ 0.05 Mean			empty plate

Experiments with full vials of substrate

Experiment 4

Repeat of experiment 3

- 250 mg of substrate powder was dissolved in 74 ml HNa-DMSO10%-solution under stirring at room temperature (magnetic stirrer)
- After the substrate fully dissolved 6 ml of 1M $CaCl_2$-solution was added. White cloudy precipitate was visible. (Documentation with photo and measurement of the Absorbance at 405 nm – plate2 well B2 – B7)
- The solution was warmed up 15 min at 37°C and dissolved with following stirring for 45 min at room temperature. (Documentation with a photo of the clear solution)
- Absorbance measurement of the solution at 405 nm after a standing time of ~ 10 minutes the solution became opal. (Documentation with a photo)

The absorbance measurement are summarized in Table 5

Table 5. Absorbance of the dissolved substrate at different time-points

Results of Absorbance measurement				
well	Abs 405nm			sample description
B2 – B4	0.041	0.037	0.037	HNaDMSO10%-Buffer
B5 – B7	0.921	0.625	0.574	Substrate + HNaDMSO10%-Buffer with $CaCl_2$
B8	0.048			empty plate

Figure 2 shows the formation of precipitates at the different stages of the experiment.

Experiments with full vials of substrate

Figure 2. Precipitate formation in TGA-Substrate – full vial

- Fluorescence Thrombin substrate dissolved in HNa – 10% DMSO solution with stirring



- Upon addition of CaCl2 a trancient cloud-like pellet formed, which disappeared after the continuous stirring and warming at 37°C



- Upon storing the solution to room temperature (required for the further procedure) within 0.5 to 120 minutes the clear solution became opal, and formed a stable precipitate, which can only be dissolved after a long-lasting vigorous shaking.



→ All these experiments showed that the fluorescence thrombin substrate can be fully dissolved in a 10 % DMSO- containing solution, however a precipitate formed upon the addition of $CaCl_2$, which can only be resolved by warming and vigorous shaking .

Fluorescence Substrate for TGA **Protocol of Dissolving Experiment**

Experiment full vial of substrate dissolved in water

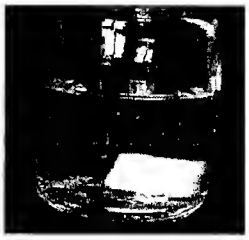
Experiment 5

According to the manufacturers instruction the Z-Gly-Gly-Arg.AMC.HCl fluorescence substrate has to be dissolved in 10% DMSO containing water. The aim of this experiment was to investigate the solubility and the effect of $CaCl_2$ addition in pure water (without DMSO). The results are summarized in Figure 3.

Figure 3. Precipitate formation in TGA-Substrate – dissolved in water without DMSO

- 1 vial of fluorescence substrate Z-Gly-Gly-Arg.AMC.HCl (250 mg, Cat. Nr. I-1140, # 0557036) was reconstituted with 74 ml of water for injection, at room temperature

- A "sticky clump" has been formed after the addition of the water The substrate could not be fully dissolved even after vigorous stirring (magnetic stirrer) for 30 minutes. An apparent clear solution with very fine precipitates appeared.



- However, the substrate did not fully dissolved, 6 ml of 1M $CaCl_2$-solution was added.
- First a white cloudy precipitate was visible, which turned to a massive fine precipitate
- No precipitate was detected in a control experiment, where $CaCl_2$ has been added to water in the absence of the substrate (No photo shown)
- The solution was warmed up to 37°C and stirred for 60 minutes
- The solution cleared up, but the fine precipitate converted to visible, bigger precipitates, which could not be solubilized et al.

→ The experiment showed that the fluorescence thrombin substrate cannot be fully dissolved in an aqueous solution, and the precipitate formed upon the addition of $CaCl_2$ cannot be resolved

Appendix

Materials, solutions and equipments

Fluorescence substrate:
Z-Gly-Gly-Arg.AMC .HCl, BACHEM Cat. Nr. I-1140, # 0557036, 250mg/vial (no Exp. Date)

Aqua dest.
sterile water for injection, Baxter, #V001321

HNa-buffer:
25 nM Hepes
175 mM NaCl
pH 7.35
sterile-filtrated (prod BK/05.09.2006)

DMSO
Fa. Merck (Dimethylsulfoxide p.a.) Cat. Nr: 2931

HNa-DMSO10%-solution:
90ml HNa-buffer + 10 ml DMSO p.a.

$CaCl_2$-solution 1M
$CaCl_2.2H_2O$: 14.7 g/100 ml a.d. (prod BK/ 05.02.2007)

Reader Synergie HT Biotek
Endpoint-measurement at 405nm
in a Nunc clear, flat bottom 96 well plate